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Delta and Egfr expression are regulated by Importin-7/Moleskin in *Drosophila* wing development

Alysia D. Vrailas-Mortimer [a,1], Neena Majumdar [b,1], Ginnene Middleton [b], Evan M. Cooke [b], Daniel R. Marenda [b,*]

[a] *Department of Cell Biology, Emory University School of Medicine, Atlanta, GA 30322, USA*
[b] *Department of Biological Sciences, University of the Sciences in Philadelphia, STC 271, 600 S. 43rd St., Philadelphia, PA 19104, USA*

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Abstract

Drosophila DIM-7 (encoded by the *moleskin* gene, *msk*) is the orthologue of vertebrate Importin-7. Both Importin-7 and Msk/DIM-7 function as nuclear import cofactors, and have been implicated in the control of multiple signal transduction pathways, including the direct nuclear import of the activated (phosphorylated) form of MAP kinase. We performed two genetic deficiency screens to identify deficiencies that similarly modified Msk overexpression phenotypes in both eyes and wings. We identified 11 total deficiencies, one of which removes the *Delta* locus. In this report, we show that *Delta* loss-of-function alleles dominantly suppress Msk gain-of-function phenotypes in the developing wing. We find that Msk overexpression increases both Delta protein expression and *Delta* transcription, though Msk expression alone is not sufficient to activate Delta protein function. We also find that Msk overexpression increases Egfr protein levels, and that *msk* gene function is required for proper Egfr expression in both developing wings and eyes. These results indicate a novel function for Msk in Egfr expression. We discuss the implications of these data with respect to the integration of Egfr and Delta/Notch signaling, specifically through the control of MAP kinase subcellular localization.

Keywords: *Drosophila*; *Moleskin*; Importin-7; DIM-7; Wing; Delta; Notch; Egfr; MAPK; dpERK

Introduction

A striking fact of development is that all multi-cellular animals develop from a single cell. It is the burden of this one cell, and all subsequent cells derived from this cell, to coordinate and control a large number of diverse cellular processes as they develop, in order to properly form a viable, fully functional organism. So important is the success of this basic developmental progression, that mis-regulation of many of these basic processes is a contributing factor to human disease. However, even with the diverse array of cellular processes required for proper development to occur, only a comparatively small number of developmental signaling pathways are required to control development. Very often, different signaling pathways intersect to regulate common cellular processes. Understanding how these signals are properly integrated during development to regulate specific cellular processes is an area of intense interest, as we are just recently beginning to understand the different mechanisms involved in such interactions.

The Ras/MAPK and Delta/Notch pathways exemplify two highly conserved and very well studied signaling pathways that can both cooperate with and antagonize each other's functions (Doroquez and Rebay, 2006; Hasson and Paroush, 2006; Sundaram, 2005; Vivekanand and Rebay, 2006). Signaling from the Ras/MAPK pathway begins at the plasma membrane with the activation of a transmembrane receptor tyrosine kinase (RTK, such as the epidermal growth factor receptor, Egfr) in response to a variety of physiological stimuli. This activation leads to RTK dimerization and autophosphorylation, resulting in the activation of the small GTPase protein Ras (Mitin et al., 2005). Ras then transduces the signal through a series of

* Corresponding author. Fax: +1 215 596 8710.
E-mail address: d.marend@usip.edu (D.R. Marenda).
[1] Equal contribution.

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phosphorylation events, ultimately leading to the dual phosphorylation and activation of MAPK (MAPK to di-phospho-MAPK, or dpERK/pMAPK) in the cytoplasm (Schaeffer and Weber, 1999; Shilo, 2003; Simon, 2000). Upon activation by phosphorylation, MAPK proteins dimerize (Cobb and Goldsmith, 2000), and can then phosphorylate targets in the cytoplasm, and/or rapidly translocate to the nucleus where they phosphorylate and regulate nuclear target proteins and gene expression (Brunet et al., 1999; Chen et al., 1992; Khokhlatchev et al., 1998; Lenormand et al., 1993).

Notch is a transmembrane receptor that is activated upon the short-range binding of the DSL (Delta/Serrate/LAG-2) family of ligands (Artavanis-Tsakonas et al., 1999; Schweisguth, 2004). Upon ligand binding, Notch is proteolytically cleaved, and the C-terminal fragment ($Notch^{intra}$) translocates to the nucleus where it can regulate nuclear target proteins and gene expression (Baron, 2003; Lai, 2004; Mumm and Kopan, 2000).

The correct subcellular localization of the components within these signaling pathways, particularly inducible transcription factors, is a key regulatory mechanism by which a cell can control the expression of signaling information, and can also serve as a point of integration between two distinct pathways (Doroquez and Rebay, 2006; Hasson and Paroush, 2006; Poon and Jans, 2005; Sundaram, 2005). Regulation of the subcellular localization of target proteins between the cytoplasm and the nucleus is accomplished by the karyopherins, a family of nuclear import and export factors (the importins and exportins respectively). Importins and exportins regulate the directional movement of targets through the nuclear pore complex (a large multi-protein complex embedded within the nuclear membrane), by utilizing the GDP/GTP-bound state of Ran GTPase (Pemberton and Paschal, 2005) (Gorlich, 1998; Görlich, 1997).

One component that functions in the regulation of the subcellular localization of many proteins is Msk/DIM-7. Msk/DIM-7 (hereafter referred to as Msk) is encoded by the *moleskin* (*msk*) gene and is the *Drosophila* homolog of Importin-7 (Lorenzen et al., 2001). In mammals, Importin-7 functions in the regulation of different cellular processes by the nuclear import of a number of proteins, including histone H1 and HIV reverse transcription complexes (Fassati et al., 2003; Jäkel et al., 1999). In flies, Msk genetically interacts with integrins (Baker et al., 2002) and with the conserved transcription factor Senseless (Pepple et al., 2007). Msk functions in the nuclear import of the homeobox gene *Caudal* (Han et al., 2004), and is a nuclear import cofactor for phosphorylated (activated) MAP kinase (pMAPK) (Lorenzen et al., 2001). Msk mediated nuclear import of pMAPK is critical for cell proliferation in the developing *Drosophila* wing (Marenda et al., 2006), and is also required for proper ommatidial rotation in the developing eye (Vrailas et al., 2006), as well as in R8 development posterior to the morphogenetic furrow with the eye as well (Pepple et al., 2007). Further, apical sequestration (and thus functional inactivation) of Msk mediates the cytoplasmic hold of pMAPK in the morphogenetic furrow of the developing eye, an event that is crucial for proper eye development (Kumar et al., 2003; Vrailas et al., 2006). Thus, a better understanding of how and in what cellular processes Msk functions may shed light on the regulation of these cellular functions, as well as the mechanisms of integration between distinct developmental signaling pathways.

In order to better understand Msk function during *Drosophila* development, we undertook a genetic deficiency screen based on the overexpression of Msk in both eyes and wings. We looked for deficiencies that similarly genetically modified the Msk phenotype in both tissues, and report here the identification of 11 such deficiencies, one of which removes the Notch ligand *Delta*. We report that loss-of-function mutations in *Delta* dominantly suppress gain-of-function Msk phenotypes in the developing wing, and that both Delta protein expression and *Delta* transcription are increased in Msk gain-of-function wings, though this Delta protein is not competent to promote Notch signaling in adjacent cells. We also report that proper Msk function is both necessary and sufficient for Egfr protein expression in developing eyes and wings. We show that where Egfr protein levels are reduced, both Dl expression and cytoplasmic pMAPK expression are increased. Conversely, where Egfr protein levels are increased, nuclear MAPK expression is also increased. Overexpression of Dl has no effect on Egfr protein levels, but does increase pMAPK expression levels. We suggest that the subcellular localization of MAPK in the developing wing plays an important role in Egfr protein expression, and that this expression in turn significantly affects both Delta protein expression and signaling competence.

Materials and methods

Drosophila stocks and culture

All stocks were crossed and maintained on standard cornmeal/molasses media at 25 °C unless otherwise indicated. Stocks used were: *en:GAL4* (gift from Ruth Palmer), *GMR:GAL4* (Hay et al., 1994; Moses et al., 1989), *hs:MG* (*p[w+, hsp70*: :*Rl-GAL4/VP16]*) (Kumar et al., 2003; Marenda et al., 2006), *hs:msk* (Vrailas et al., 2006), *UAS:GFP* (Bloomington Stock Center,http://flybase.bio.indiana.edu/), *UAS:msk*, msk^5 (Lorenzen et al., 2001), Dl^{RF} (Parks and Muskavitch, 1993), Dl^{B2} (Micchelli et al., 1997), *UAS:Dl* (gift from Gary Struhl), *wg-lacZ* (Kassis, 1990), *eyFLP* (Newsome et al., 2000), $Dl^{S111909}$ (*Dl-lacZ*, Szeged Stock Center,http://expbio.bio.u-szeged.hu/fly/index.php). To make *msk* clones in the eye, virgin female *eyFLP*; *FRT 80B*, *Ubi-GFP*/TM6B flies were crossed to male w^-; *FRT 80B* msk^5/TM6B flies. For *msk* clones in the wing, virgin female y^-,w^-, *hsFLP; M(3)55 PUB nuc-GFP(34C3) FRT80B/TM6* (a gift from Mathew Freeman) were crossed to male w^-; *FRT 80B* msk^5/TM6B flies, and heat shocks (at 37 °C) were administered for 1 h during the first larval instar stage.

Deficiency screen

Prior to initializing the screen, heterozygous *en:GAL4*, *UAS:msk* and *GMR:GAL4*, *UAS:msk* stocks were isogenized for the second and third chromosome. Virgin females of each stock were then crossed to males from each Bloomington deficiency stock (http://flystocks.bio.indiana.edu/Browse/df-dp/dfkit.htm) and F1 progeny were analyzed for genetic interaction in eyes or wings as appropriate.

Immunohistochemistry, Western blotting, and tissue mounting

Wing disc and eye disc preparations were as described (Tio and Moses, 1997), mounted in Vectashield (Vector Labs, H-1000), and imaged by confocal microscopy. Primary antibodies: rabbit anti-beta-galactosidase (1:1000, Cortex Biochem CA2190), mouse anti-Delta (1:50, Iowa Hybridoma Bank #C594.B9), mouse anti-Notch (1:200, Iowa Hybridoma Bank #C17.9C6), mouse anti-cut (1:10, Iowa Hybridoma Bank #2B10), rabbit anti-Egfr (Rodrigues et al., 2005),

and mouse anti-pMAPK (1:500, Sigma). Secondary antibodies were from Jackson ImmunoResearch: goat anti-mouse Cy5 (1:500, 115-175-003), goat anti-rabbit TRITC (1:250, 111-025-003).

Western blots were performed as described (Dingwall et al., 1995). Primary antibodies used were: rabbit anti-Egfr (Rodrigues et al., 2005), and goat anti-tubulin (1:2000). Secondary HRP-conjugated antibodies were: goat anti-rabbit HRP (1:8000, Santa Cruz Biotechnology) and rabbit anti-goat HRP (1:50,000, Santa Cruz Biotechnology).

Adult wings were dehydrated in ethanol, mounted in DPX (Zeiss), and photographed. Adult eyes were immersed in ethanol and photographed.

Results

Msk overexpression disrupts eye and wing development

To overexpress Msk in eyes and wings we used *UAS:msk* (Lorenzen et al., 2001) driven by either *GMR:GAL4*, which expresses the GAL4 protein posterior to the morphogenetic furrow in the developing eye, or *en:GAL4*, which expresses the GAL4 protein in the posterior compartment boundary in the developing wing.

Yeast GAL4 protein activity is temperature sensitive, and we used this aspect of the protein to control the level of Msk overexpression during our analysis. Expression of *GMR:GAL4* at 18 °C had no effect on the morphology or development of the *Drosophila* eye (Fig. 1A), showing both normal sized and properly pigmented eyes. However, expression of *UAS:msk* with *GMR:GAL4* (*GMR*∷*msk*) at 18 °C showed both a slight roughening of the eye, as well as loss of red pigment (Fig. 1B). This effect is enhanced at 25 °C (Fig. 1C), and is lethal at 29 °C.

Similarly, expression of *en:GAL4* at either 18 °C (data not shown) or 25 °C (Fig. 2A) had no effect on the morphology or development of the *Drosophila* wing, showing both normally sized wings with properly formed and spaced vein and bristle tissue. Expression of *UAS:msk* with *en:GAL4* (*en*∷*msk*) also showed little to no phenotype at 18 °C (Fig. 2B), but showed a disrupted posterior wing compartment at 25 °C with loss of vein structures and wing tissue (Fig. 2C). As cell death is induced in wings of this genotype (Marenda et al., 2006), the phenotype is most likely related to tissue loss due to increased apoptosis. As in the developing eye, this genotype is also lethal at 29 °C.

As overexpression of Msk at 25 °C in both eyes and wings gave an intermediate phenotype in each tissue, we used this temperature to perform an autosomal genetic modifier screen using the Bloomington Stock Collection Deficiency kit. In this screen we looked for deficiencies that could similarly dominantly modify the Msk overexpression phenotype in both tissues. We identified a total of 11 Bloomington deficiencies that dominantly modified both the *GMR*∷*msk* phenotype in the adult eye and the *en*∷*msk* phenotype in the adult wing in the same way (Table 1).

Deficiencies that suppressed *GMR*∷*msk* at 25 °C showed increased eye pigmentation, though eye morphology remained disrupted (Figs. 1E, F). Conversely, deficiencies that enhanced *GMR*∷*msk* at 25 °C showed a more severe disruption of eye morphology, often with a Bar-like phenotype, and displayed large patches of necrotic tissue throughout the eye field (Figs. 1G, H).

In wings, deficiencies that suppressed *en*∷*msk* showed a larger posterior wing compartment along with recovery of wing

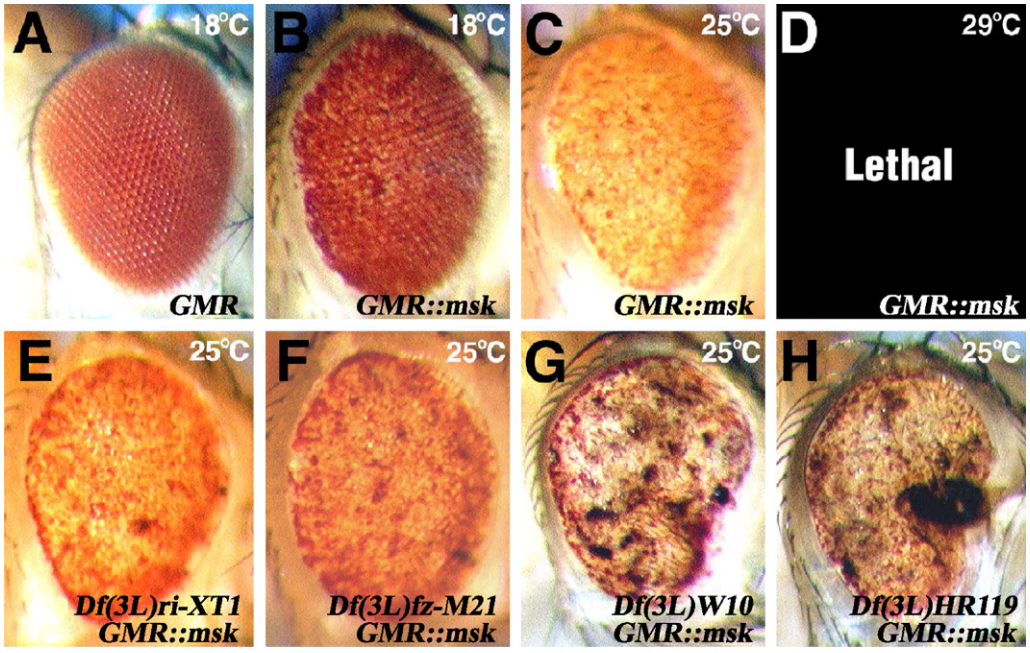

Fig. 1. Msk overexpression affects eye development. Panels A–H show adult eyes, anterior right, dorsal up, genotypes indicated bottom right. Temperatures raised are indicated top right. (A) *GMR:GAL4* alone shows phenotypically wild-type eye at lower temperatures. (B–D) Eye phenotypes of Msk overexpression posterior to the morphogenetic furrow (*GMR*∷*msk*) at increasing temperatures. (E, F) Suppression of *GMR*∷*msk* eye phenotype at 25 °C by loss of indicated genomic deficiencies. (G, H) Enhancement of *GMR*∷*msk* eye phenotype at 25 °C by loss of indicated genomic deficiencies.

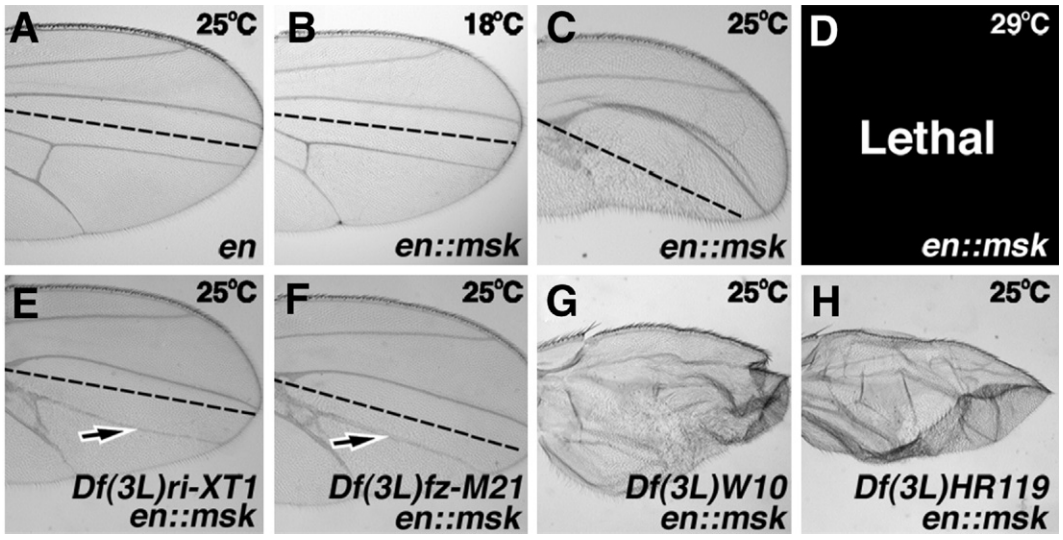

Fig. 2. Msk overexpression affects wing development. Panels A–H show adult wings, anterior up, distal right, genotypes indicated bottom right. Dotted lines in each panel represent the anterior/posterior (A/P) boundary. Temperatures raised are indicated top right. (A) *en:GAL4* alone shows phenotypically wild-type wing at lower temperatures. (B–D) Adult wing phenotypes of Msk overexpression within posterior compartments of developing wings (*en*::*msk*) at increasing temperatures. (E, F) Suppression of *en*::*msk* wing phenotype at 25 °C by loss of indicated genomic deficiencies. Arrows indicate rescue of L4 vein. (G, H) Enhancement of *en*::*msk* wing phenotype at 25 °C by loss of indicated genomic deficiencies.

vein L4 specification (Fig. 2, arrows in E, F), while deficiencies that enhanced the *en*::*msk* phenotype displayed smaller posterior wing compartments, increased vein loss, and an overall decrease in normal wing morphology (Figs. 2G, H).

Loss-of-function Delta mutants suppress Msk overexpression phenotypes in the wing

Among the deficiencies that suppress the *en*::*msk* wing phenotype was *Df(3R)DL-BX12*. While trans-heterozygous *Df(3R)DL-BX12/GMR*::*msk* flies were lethal at 25 °C, *Df(3R)DL-BX12/en*::*msk* flies displayed a suppression of the *en*::*msk* wing phenotype, suppressing both the small posterior wing compartment size, as well as the loss of wing vein L4 specification (compare Fig. 3B to Fig. 2C; Table 1). One gene deleted within this deficiency region is *Delta* (*Dl*), which encodes a transmembrane ligand for the *Notch* (*N*) signal transduction pathway (Artavanis-Tsakonas et al., 1995, 1999; Muskavitch, 1994; Parody and Muskavitch, 1993). We therefore tested whether alleles of *Dl* could also dominantly suppress the *en*::*msk* wing phenotype. We tested two different loss-of-function alleles of *Delta*, Dl^{RF} and Dl^{B2}. Both showed a suppression of the *en*::*msk* gain-of-function wing phenotype similar to that observed with the *Df(3R)DL-BX12* deficiency (Figs. 3C, D). These data suggest that it is loss of function at the *Delta* locus within this deficiency that is responsible for the suppression of the *en*::*msk* phenotype observed with *Df(3R)DL-BX12*.

Because loss-of-function *Dl* alleles suppressed the Msk gain-of-function phenotype in the wing, we next examined whether Msk gain-of-function could increase Dl expression or function in the developing larval wing. Dl protein is normally expressed in both wing margin and pro-vein cells in the late third instar larval wing pouch (Figs. 3E, F) (de Celis and Bray, 1997; Kooh and Muskavitch, 1993; Micchelli et al., 1997). Overexpression of Msk in the posterior compartment of the wing pouch causes a dramatic increase in Dl protein expression in this compartment (Figs. 3G, H). This increased Dl protein expression could be a result of reduced Dl degradation, altered Dl subcellular localization, or increased *Dl* transcription within these cells. To further investigate this aspect of Dl regulation, we utilized a *Delta* enhancer-trap fly line ($Dl^{S111909}$) (Salzberg et al., 1997) that expresses β-galactosidase under the control of the *Delta* enhancer, in a fashion similar to both Delta protein expression and *Delta* RNA expression (Fig. 3I) (de Celis et al., 1997). We find that β-galactosidase expression is increased in the posterior compartment of wing cells that overexpress Msk (Fig. 3J), suggesting that Msk overexpression increases *Dl* transcription in developing wing cells. To verify that our β-galactosidase antibody is not itself affected by Msk overexpression in this compartment, we also analyzed the transcriptional expression of *Wingless* by utilizing an enhancer-trap fly line (*Wg-lacZ*). *Wg* is normally expressed along the dorsal/ventral boundary in developing wing discs (Baker, 1988), and this is also where we observe *Wg* β-galactosidase expression (Fig. 3K). In *en*::*msk* wing discs, we find that *Wg* β-galactosidase expression

Table 1

Deficiency	Cytology	Eye	Wing
Df(3L)W10*	75A6-7;75C1-2	Enhance	Enhance
Df(3L)GN34	63E6-9;64A8-9	Enhance	Enhance
Df(3L)HR119	63C6;63F7	Enhance	Enhance
Df(3L)fz-M21	70D2;71E4-5	Suppress	Suppress
Df(3L)ri-XT1	77E2;78A4	Suppress	Suppress
Df(3R)by10	85D8-12;85E7-F1	Lethal	Lethal
Df(3R)M-Kx1	86C1;87B1-5	Lethal	Enhance
Df(3R)Dl-BX12	91F1-2;92D3-6	Lethal	Suppress
Df(2R)en-A	47D3;48B2	Lethal	Lethal
Df(2L)BSC37	22D2-3;22F1-2	Lethal	Lethal
Df(2R)en30	48A3-4;48C6-8	Lethal	Enhance

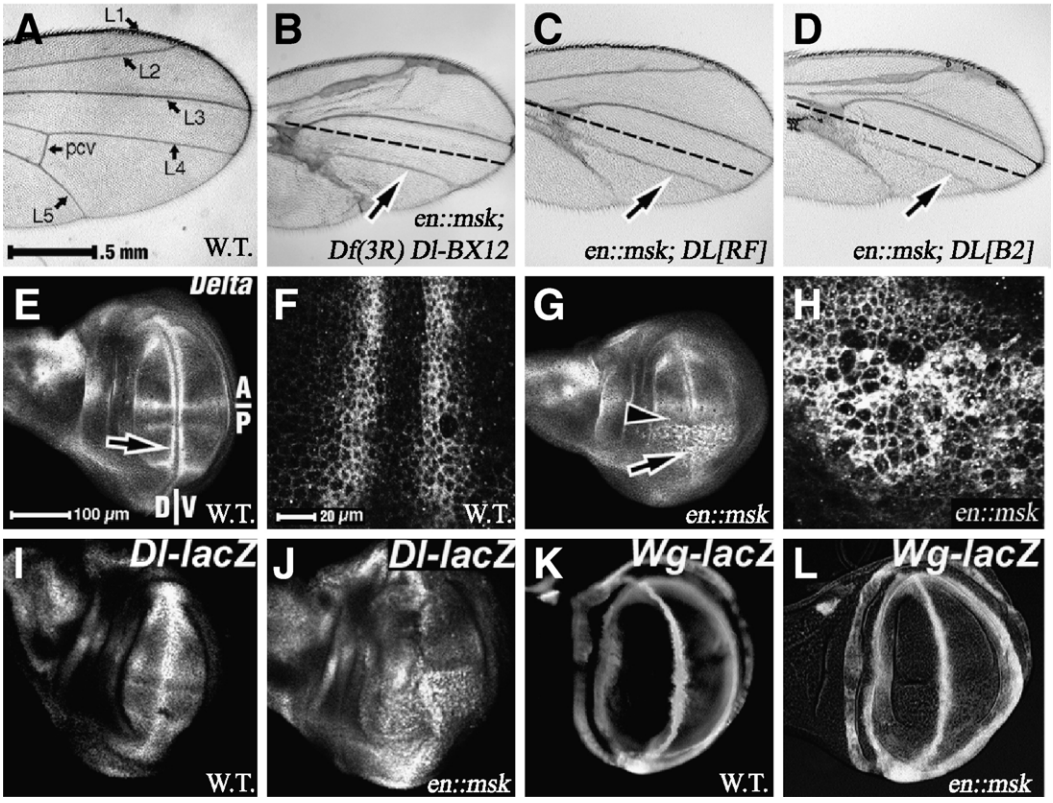

Fig. 3. Loss-of-function *Delta* mutations suppress Msk overexpression phenotypes in adult wings. (A–D) Adult wings, anterior up, distal right. Magnification is equal in panels A–D and panels E, G, I–L, and panels F and H as indicated. Genotypes indicated below right. (A) The normal venation pattern is labeled with longitudinal veins (L1–L5) and posterior cross-vein (pcv) indicated. (B) Dotted line separates anterior (top) from posterior (bottom) of the wing in this and subsequent panels. Msk overexpression is driven in the posterior compartment (*en::msk*) of genomic deficiency *Df(3R) Dl-BX12*. Arrow indicates rescue of L4 vein normally absent in *en::msk* wings. (C–D) Arrows indicate suppression of *en::msk* by loss-of-function in one copy of $Delta^{RF}$ (C), and $Delta^{B2}$ (D). (E–L) Larval wings, anterior/posterior (A/P) and dorsal/ventral (D/V) as indicated in panel E. Antigens shown indicated above right. Genotypes indicated below right. (E, F) Wild-type Delta protein expression. Panel F shows high magnification of the area at arrow in panel E. (G, H) Msk overexpression in the posterior compartment (*en::msk*). (G) Arrowhead shows A/P boundary. Arrow indicates increased Delta protein expression. Panel H shows high magnification of the area at arrow in panel G. (I–L) Anti-β-galactosidase expression in (I) *Delta-lacZ*, (J) *Delta-lacZ*, *en::msk*, (K) *Wingless-lacZ*, and (L) *Wingless-lacZ*, *en::msk* wing discs.

is normal (Fig. 3L), suggesting that our β-galactosidase antibody is itself not affected by Msk overexpression. Taken together, these results suggest that Msk gain-of-function in the developing wing is sufficient to induce *Delta* transcription and increased Dl protein expression within this tissue, and that this increased Delta expression is partly responsible for the phenotypic effects observed in Msk gain-of-function wings.

Msk function is dispensable for Delta expression or function in developing wings and eyes

Dl protein expression in the wing margin is responsible in part to activate both the expression of the homeobox gene *cut* (*ct*) as well as *Wg* expression in adjacent margin cells (Figs. 3K, 4A) (Blochlinger et al., 1993; Jack et al., 1991; Micchelli et al., 1997), and clonal overexpression of Dl in the developing wing pouch is sufficient to induce expression of both *Wg* and *ct* in adjacent cells surrounding the Dl expressing clones (de Celis and Bray, 1997; Wang and Struhl, 2004). Notch is modified in the dorsal wing compartment by the glycosyltransferase Fringe (Fng), such that Notch preferentially responds in dorsal cells to Dl signaling from ventral wing cells (Blair, 2000; de Celis and Bray, 1997; Fleming et al., 1997; Moloney et al., 2000; Panin et al., 1997). As we do not observe altered *Wg* expression in the posterior compartment of *en::msk* wing discs (Fig. 3L), this suggested that the Dl protein expression induced by Msk overexpression within the posterior wing compartment is not competent to promote Notch signaling in adjacent anterior/dorsal cells.

To further test this observation, we utilized Cut expression as a downstream reporter of Notch activation in wing discs (Wang and Struhl, 2004). We first analyzed whether overexpression of Delta itself in the posterior compartment of developing wings could ectopically activate Notch signaling in adjacent anterior/dorsal cells. Overexpression of Dl (*UAS:Dl; en:GAL4*, *UAS:GFP*, or *en::Dl*) in this fashion led to a dramatic increase in disc size (Figs. 4B, C), and was also able to induce Ct expression in adjacent dorsal wing cells (arrowhead in Fig. 4B, next to GFP marker expression in panel C). Normal Ct expression in the posterior compartment of these discs, however, was eliminated (arrow in Fig. 4B). These results are consistent with previously reported cases where high levels of Dl protein within Dl overexpression clones autonomously inhibits Notch activation within the clone (de Celis and Bray, 1997; Micchelli et al., 1997;

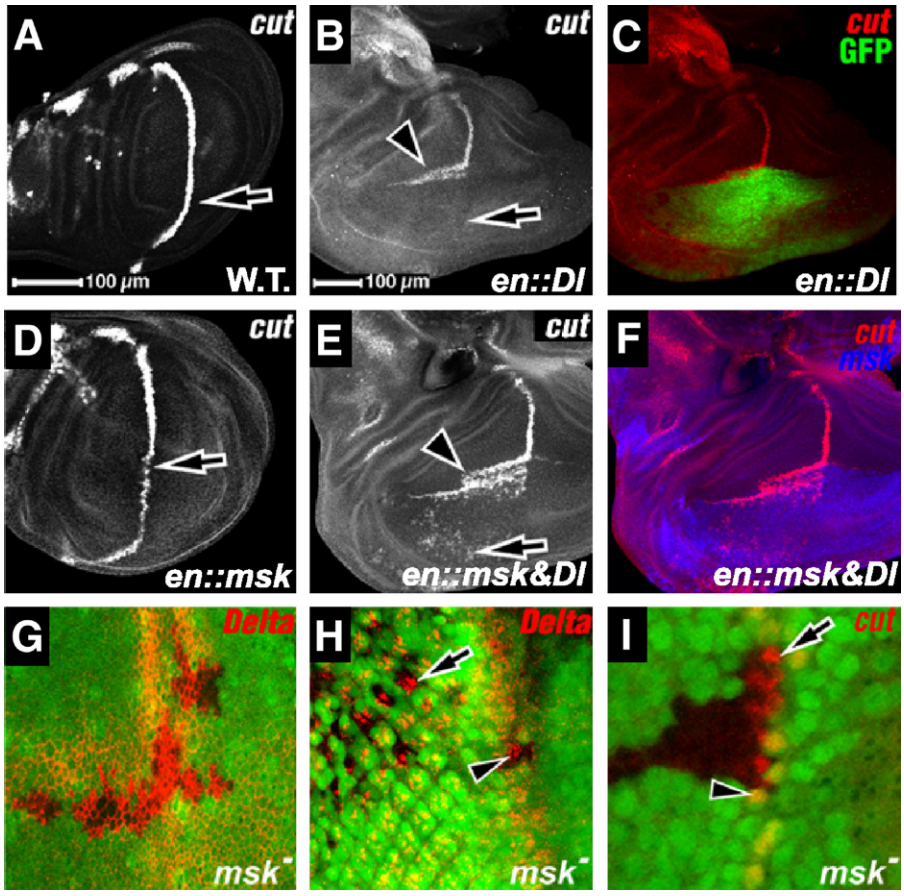

Fig. 4. Msk overexpression alters Dl protein expression. (A–G and I) Larval wings, anterior up, dorsal right. (H) Larval eye, anterior right. Antigens shown and GFP indicated above right. Genotypes indicated below right. (A) Wild-type Cut protein expression in larval wing. Arrow indicates posterior compartment Cut expression. (B) Delta overexpression in the posterior compartment (*en∷Dl*). Arrow indicates loss of Cut protein expression in the posterior compartment (compare to arrow in panel A). Arrowhead indicates ectopic Cut protein expression along anterior/dorsal cells. Panel C shows Delta expression (marked by posterior GFP expression) merged with ectopic Ct protein expression in adjacent anterior/dorsal cells. (D) Msk overexpression in the posterior compartment (*en∷msk*). Arrow indicates reduced Cut protein expression in the posterior compartment. (E) Overexpression of both Msk and Delta in posterior wing disc compartments (*en:GAL4* driving both *UAS: Delta* and *UAS:msk*, *en∷Dl*, *en∷msk*). Arrow shows ectopic *cut* expression in posterior/dorsal cells. Panel F shows Msk protein expression (in blue) with Cut protein expression (in red) in both anterior/dorsal cells adjacent to Msk/Dl co-expressing cells, as well as in posterior/dorsal cells not adjacent to Msk/Dl co-expressing cells. (G–I) Mosaic msk^5 null somatic clones in late third instar larval wing discs (G, I) and eye discs (H). msk^5 null clones are negatively marked with GFP (green) in all panels. (G) Delta protein expression in msk^5 null clones is not significantly altered within or outside clones in developing wings. (H) Delta protein expression in eyes with msk^5 null clones is not significantly altered within or outside clones either within the morphogenetic furrow (arrowhead) or posterior to the morphogenetic furrow (arrow). (I) Cut protein expression in wings with msk^5 null clones is not significantly altered within (arrow) or outside (arrowhead) the clones. Magnification is equal in panels A and D, panels B, C, E, F, and panels G, H as indicated.

Wang and Struhl, 2004; Wang and Struhl, 2005). The results also suggest that overexpression of Dl alone within the posterior compartment of the developing wing is sufficient to promote Notch signaling (as measured by Ct expression) in anterior/dorsal cells adjacent to Dl expression, as expected.

We next examined Ct expression in *en∷msk* wing discs. While Ct expression is this genetic background was reduced in the posterior compartment, it was not completely lost (arrow in Fig. 4D) as is observed when we overexpress Dl alone (arrow in Fig. 4B). Moreover, there is no ectopic expression of Ct adjacent to Msk expression in anterior/dorsal cells (compare Fig. 4D to arrowhead in Fig. 4B). Taken together with our results from *Wg-lacZ* expression within this genotype, we suggest that though Msk overexpression does induce greater Dl expression within developing wing discs, this Dl is not competent to promote Notch signaling in adjacent cells. Further, as we see decreased Cut expression in *en∷msk* wing discs, Msk expression may also have a negative function on Dl activity while simultaneously increasing Dl expression. To test this, we overexpressed both Dl and Msk together in the posterior compartment of developing wing discs (*UAS:Dl; UAS:msk*, *en:GAL4*, or *en∷msk* and *Dl*). We find that posterior cells expressing both Msk and Dl are still able to signal to adjacent anterior/dorsal cells (arrowhead in Figs. 4E and F). However, we now also observe interspersed Cut activation throughout the posterior/dorsal compartment of these wing discs (arrow in Fig. 4E). These data suggest that though co-expression of Msk with Dl does not affect the ability of the expressed Dl protein to signal to adjacent cells, it does in some way reduce the inhibitory effect that high levels of expressed Dl protein has on Notch signaling within the posterior compartment. Interestingly, we also observe increased Notch protein expression in the posterior compartment of *en∷msk* wings

(Supplemental Figure 1), which may also have an inhibitory effect on expressed Dl within this compartment (discussed below).

Because overexpression of Msk induces Dl transcription, and also affects downstream Notch activation (as measured by reduced Cut expression), we sought to determine if *msk* gene function was necessary for Delta expression in developing fly tissues. We generated *msk* mosaic null clones (*msk*^{5}) (Baker et al., 2002; Lorenzen et al., 2001; Vrailas et al., 2006) and analyzed expression of both Delta and Cut in developing wing and eye tissue.

msk Function is normally required for cell survival in both the developing eye and wing tissues (Baker et al., 2002; Lorenzen et al., 2001; Vrailas et al., 2006), and *msk* null clones are rare when induced with heat shock (*hs:Flp*). To overcome this obstacle in developing wings, we utilized the *Minute* technique (Lawrence et al., 1979; Morata and Ripoll, 1975; Xu and Rubin, 1993), which confers upon *msk* null clones a growth advantage. In the developing wing disc, Dl expression is not lost in *msk* null clones (Fig. 4G), nor is Ct expression (arrow in Fig. 4I), suggesting that *msk* gene function is not required for either proper Delta expression or Notch activity within this tissue. Similar results were observed with Notch protein expression within *msk* clones (Supplemental Figure 1) within this tissue. However, it has been shown that using *Minute* chromosomes can non-autonomously affect gene expression in developing mutant or wild-type clones (de la Cova et al., 2004; Rodrigues et al., 2005), which may lead to artifacts in interpretation of results. To address this issue, we generated *msk* null clones in a non-*Minute* background utilizing the developing fly eye (Vrailas et al., 2006). We similarly found that Dl expression within *msk* clones in late third instar eye discs was normal both within and posterior to the morphogenetic furrow (arrowhead and arrows in Fig. 4H), as was Notch expression (Supplemental Figure 1). Taken together, these results suggest that *msk* is dispensable for both Dl and Notch expression in eyes and wings, and Ct expression in wings.

Msk is necessary and sufficient for Egfr expression in developing wings and eyes

Because *msk* null clones have no discernable effect on either Dl, N, or Ct expression in developing wings, Msk gain-of-function effects on Delta and Notch expression may be an indirect result of Msk overexpression on a different pathway. A prime candidate for this pathway is the Egfr/Ras/MAPK pathway. The *Drosophila* Msk/DIM-7 protein functions as a nuclear import cofactor for the phosphorylated (activated) form of MAP kinase (Lorenzen et al., 2001). In the developing wing, overexpression of DIM-7 in the posterior compartment leads to increased nuclear translocation of MAPK, the major downstream effector of Egfr signaling in this tissue (Marenda et al., 2006). Overexpression of activated Egfr protein leads to ectopic Dl expression in the developing wing (Tsuda et al., 2002), and in developing cone cells of the larval and pupal eye (Nagaraj and Banerjee, 2007). Since Egfr is the receptor tyrosine kinase that activates Ras/MAPK signaling in developing wings (Guichard et al., 1999; Shilo, 2003; Shilo, 2005), and we observe an increase in Dl expression in *en::msk* wing discs, we sought to test whether this effect on Dl might be indirectly mediated by Msk through alterations in Egfr protein expression.

Egfr mRNA is broadly expressed in larval wings, but it is downregulated in wing margin and vein territories (Guichard et al., 1999). Using an antibody specific to Egfr (Rodrigues et al., 2005), we observed similar regulation of Egfr protein expression in larval wings, with broad expression of Egfr protein throughout the tissue that is downregulated in the presumptive wing margin and vein territories (brackets in Figs. 5A, B). However, in *en::msk* wing discs, we observe increased expression of Egfr protein within the wing margin of the posterior domain of the wing pouch when compared to the Egfr expression within the wing margin of the anterior domain of the wing pouch (brackets in Figs. 5C, D), suggesting that Msk expression is sufficient to induce increased Egfr expression within this tissue in areas where Egfr expression is normally lower.

To examine loss-of-function effects of *msk* on Egfr expression, we next examined Egfr expression in *msk* null clones. As before, we used the Minute technique to confer upon *msk* null clones a growth advantage. We observed that in developing wings, Egfr expression is reduced within *msk* null clones (Figs. 5E, F and I, J), suggesting that *msk* function is required for proper Egfr expression in this tissue. As before, we generated *msk* null clones in a non-*Minute* background utilizing the developing fly eye to verify our results. In the developing eye disc, high level of Egfr expression is normally seen within the morphogenetic furrow (Lesokhin et al., 1999; Rodrigues et al., 2005). As in the developing wing, we also observed reduced Egfr expression in *msk* null clones within the morphogenetic furrow of developing eye discs (Figs. 5G, H and K, L). Taken together, our results suggest that *msk* function is both necessary and sufficient for Egfr expression in the developing wing.

Msk overexpression in *en::msk* wing discs is constitutive and is limited to the posterior compartment of wing discs. However, our data from the developing eye suggest that Msk may be broadly required in many tissues for Egfr expression. To further investigate the relationship between Msk and Egfr expression, we analyzed whole larvae Egfr protein expression after transient expression of Msk under the control of the heat shock promoter, *hs:msk* (Vrailas et al., 2006). We induced the expression of *hs:msk* for 1 h at 37 °C, and analyzed Egfr expression in whole larvae at various times after recovery of this induction. As a control, we also analyzed Egfr expression in whole larval extracts of wild-type flies after a similar 1-h heat shock. Neither genotype showed any difference in Egfr protein expression without heat shock stimulation (Figs. 6A, B). Similarly, in both wild-type and *hs:msk* whole larval extracts, when we dissected larvae immediately after the 1-h heat shock induction (0 h after stimulation), we do not observe any significant increase in Egfr protein expression in either genotype (lanes 0 in Figs. 6A, B). However, Egfr levels are increased in *hs:msk* flies after 1 h of induction that is followed by a 1-h recovery period (compare lanes 1 in Figs. 6A, B). This up-

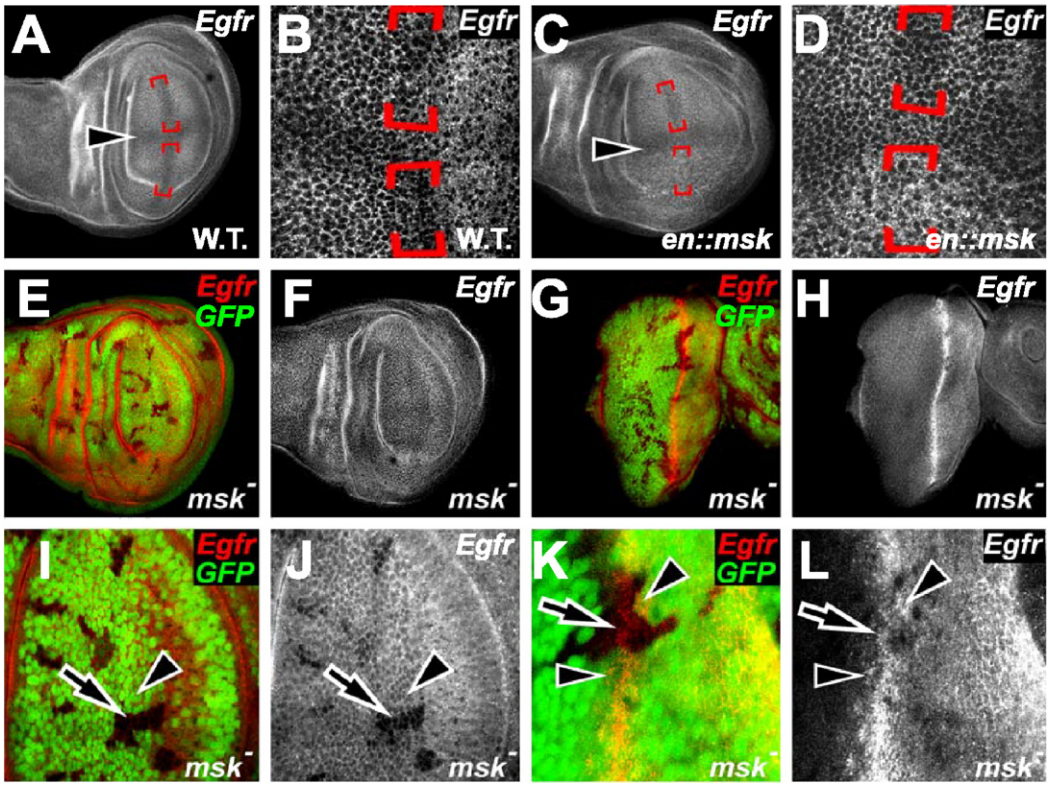

Fig. 5. Egfr expression is altered in *msk* gain- and loss-of-function. Panels show late third instar larval wings (A– D, E, F, I, J) anterior up, dorsal left, and eyes (G, H and K, L) anterior right. (A, B) Normal Egfr protein expression in wing discs. (A) Arrowhead denotes the anterior/posterior compartment boundary. Panel B shows high magnification of the area at arrowhead in panel A. Panels C, D show Egfr expression in *en::msk* wing discs. (C) Arrowhead denotes the anterior/posterior boundary. Msk expression occurs in the posterior compartment. Note the increased Egfr protein expression within this compartment. Panel D shows high magnification of the area at arrowhead in panel C. (E–L) All panels show msk^5 null clones marked by the absence of GFP expression (green) within the tissue. All panels show Egfr protein expression (red or white as appropriate) within tissues. Note the decreased Egfr expression within clones in the developing wing (E, F) and eyes (G, H). Panels I, J show high magnification of clones in developing wings. Note Egfr protein expression is decreased within clones (arrow) as compared to outside clones (arrowhead). Panels K, L show high magnification of clones in developing eyes. Note Egfr protein expression is decreased within clones (arrow) as compared to outside clones (arrowhead).

regulation is temporary, after 2-h recovery after induction, Egfr levels return to normal (compare lanes 2 in Figs. 6A, B). Msk functional protein levels produced by *hs:msk* are transient (Vrailas et al., 2006), consistent with our observation here that increased Egfr protein levels induced by *hs:msk* are able to recover after time.

When combined with our previous data, these data strongly suggest that Egfr protein expression depends on the functional levels of Msk protein. Further, this effect is not immediate, suggesting that Msk may not be directly affecting Egfr protein levels, but may rather have an indirect effect, increasing Egfr expression as part of a positive feedback mechanism after pMAPK is moved into the nucleus of appropriate cells for example (see discussion below).

Dl protein expression correlates with decreased Egfr expression and increased pMAPK expression

In order to determine the spatiotemporal relationship between Delta, Egfr, and MAPK expression, we analyzed the expression of each component in developing third instar wings. Dl protein expression mimics the expression of the phosphorylated version of MAP kinase (pMAPK) at this time (Figs. 7A, B) (Marenda et al., 2006; Tsuda et al., 2002). Interestingly, where we observe increased levels of pMAPK, we also observe decreased levels of Egfr (Figs. 7B, C, and F). pMAPK expression in developing wing discs is predominantly cytoplasmic (Marenda et al., 2006), so to determine the relationship between nuclear directed MAPK

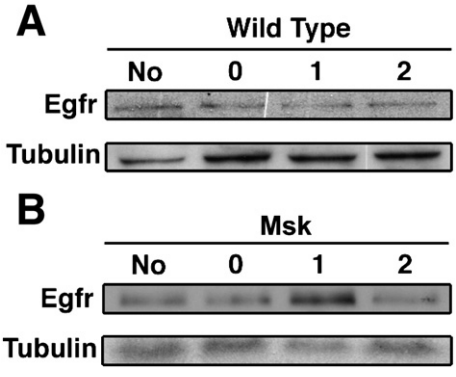

Fig. 6. Upregulation of Egfr by Msk is transient. Western blot of wild-type and *hs:msk* whole larval extracts probed with anti-Egfr and anti-tubulin antibodies. (A) Egfr expression in wild-type larvae after no heat shock (No), a 1-h heat shock followed by no recovery (0), 1-h recovery (1) and 2 h of recovery (2). (B) Egfr expression in *hs:msk* larvae after no heat shock (No), a 1-h heat shock followed by no recovery (0), 1-h recovery (1) and 2 h of recovery (2). Note increased Egfr expression after 1 h of recovery. Tubulin is protein loading control.

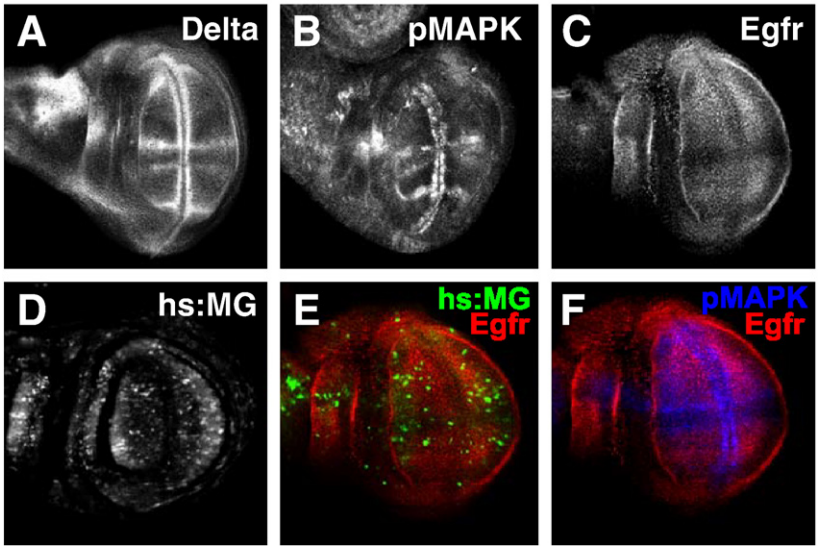

Fig. 7. Delta and Egfr expressions are specific to MAPK subcellular localization. All panels show wild-type late third instar larval wings, anterior up, dorsal left. (A) Delta expression in developing wings. (B) pMAPK expression in developing wings. (C) Egfr expression in developing wings. (D) MAPK/GAL4 (MG)-driven GFP expression to show nuclear MAPK in developing wings. (E) Egfr (red) co-localized with MAPK/GAL4 (MG)-driven GFP (green). (F) Egfr (red) co-localized with pMAPK expression (blue).

and Egfr expression, we utilized a MAPK-GAL4 fusion protein that turns on reporter gene expression (GFP) only if it reaches the nucleus of cells, as described (Kumar et al., 2003; Marenda et al., 2006). We observe that where MAPK does enter the nucleus (Fig. 7D), we observe high levels of Egfr protein expression (Fig. 7E). Thus, where Dl protein expression levels are high, Egfr protein expression is low, and cytoplasmic pMAPK levels are high. Where Dl expression is absent, Egfr expression is high, and nuclear MAPK expression is also increased.

To further examine the relationship between Dl, Egfr, and pMAPK expression, we overexpressed Dl in the posterior compartment of developing wing discs (*en∷Dl*) and analyzed the expression of both pMAPK and Egfr protein levels. pMAPK expression is normally expressed in developing wing veins and margin cells within the posterior compartment (arrow in Fig. 8A). Upon Dl expression within this compartment (marked by GFP in Fig. 8B), pMAPK expression is elevated (arrow in Fig. 8C). This increased pMAPK expression does not correlate with

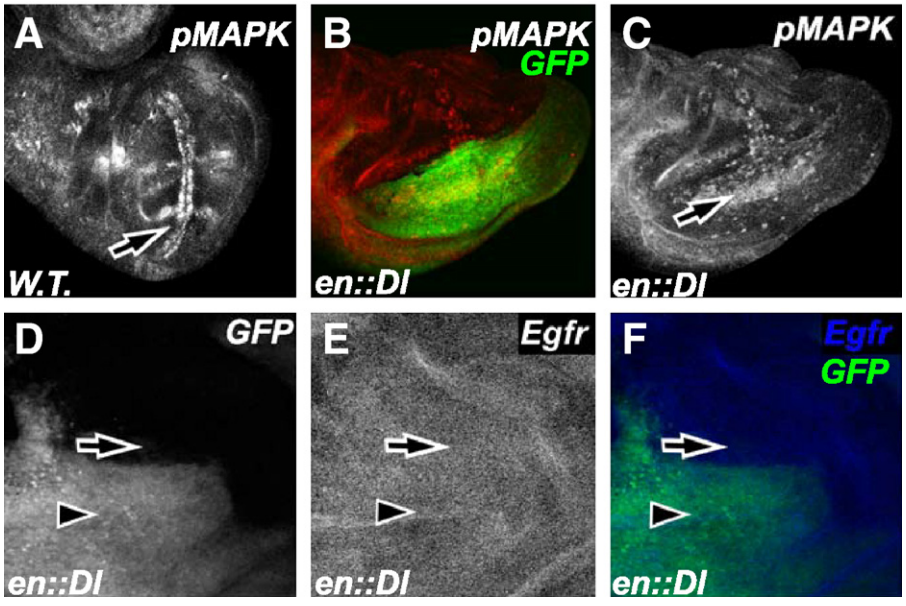

Fig. 8. Delta expression induces pMAPK expression without increasing Egfr expression. All panels show wild-type late third instar larval wings, anterior up, dorsal left. (A) pMAPK expression in a normal wing disc. Arrow indicates expression in veins and wing margin in the posterior compartment. (B–F) Delta overexpression in the posterior compartment (*en∷Dl*). GFP shows where ectopic Dl is expressed in panel B. Arrow in panel C denotes increased pMAPK expression in the posterior compartment of these discs. (D–F) Egfr expression in *en∷Dl* discs. Arrows denote anterior expression in all panels. Arrowheads denote posterior expression in all panels. Dl expression is marked by GFP in panels D and F.

an increase in Egfr protein levels. Indeed, Egfr expression within the posterior compartment of *en∷Dl* wing discs remains similar to that observed in the control anterior compartment (compare posterior arrowheads to anterior arrows in Figs. 8D–F). These data are consistent with what we have observed in wild-type discs (Fig. 7) and suggest that the subcellular localization of MAPK may play an important role in determining Egfr-mediated Dl expression and/or signaling competency (see below).

Discussion

We performed an autosomal genetic modifier deficiency screen in *Drosophila*, utilizing a Msk overexpression phenotype in both eyes and wings. With each phenotype individually, a number of deficiencies had a dominant effect. However, in an attempt to identify deficiencies that more generally affect *msk* function, as opposed to deficiencies that affect general tissue-specific morphology, we only considered those deficiencies which showed a similar effect in both Msk overexpression eyes and wings. Of the 217 deficiencies tested, only 11 dominantly modified both overexpression phenotypes in a similar manner (Table 1).

Here, we have focused our analysis on the effects of mutation in *Delta*, which was identified in this screen. We have shown that loss-of-function mutations in *Delta* dominantly suppress Msk overexpression phenotypes in developing wings. Further, we have shown that *Delta* transcription and Delta protein expression are increased in areas overexpressing Msk protein in developing wing discs. Interestingly, the increased Delta protein induced by Msk overexpression is not competent to activate Notch signaling in adjacent cells. Thus, some mechanism must either be inhibiting this induced Dl protein from signaling to adjacent cells, or the induced Dl protein itself is non-functional for signaling.

Delta must be endocytosed in signal-sending cells in order to activate Notch in signal-receiving cells (Le Borgne and Schweisguth, 2003; Parks et al., 2000; Wang and Struhl, 2005, 2004). Wang et al. report that clones of cells that express Dl but are also deficient for Epsin, an adapter protein required for Clathrin-mediated endocytosis, similarly cannot promote Notch signaling in adjacent cells (Wang and Struhl, 2004). These authors propose that the Delta protein must normally be endocytosed and mono-ubiquitinated in the signal-sending cells (Delta expressing cells), where it is then targeted to a special endocytic pathway where it acquires competency to activate Notch in signal receiving cells (Wang and Struhl, 2004; Wang and Struhl, 2005). Thus, overexpression of Msk may have some effect on the internalization and/or post-translational modification of Delta (mono-ubiquitination) to render it unable to signal to adjacent cells. Indeed, we observe that Msk protein expression in *en∷msk* wing discs is in a pattern that is co-incident with disrupted Delta protein near the apical tips of cells in the wing disc (Supplemental Figure 2). Msk expression has previously been observed in the apical tips of cells within the morphogenetic furrow in the developing eye disc (Vrailas et al., 2006), where this apical localization is proposed to functionally inactivate Msk nuclear translocation function. Thus, in *en∷msk* wing discs, apical localization of Msk protein may disrupt important cellular functions at this localization in the cell, such as Dl internalization and/or compartmentalization.

Previous reports have shown that levels of overexpressed exogenous Delta in clones of cells are several fold higher than normal peak levels of endogenous Dl protein expression, and this overexpression autonomously inhibits Notch activation within these clones (de Celis and Bray, 1997; Micchelli et al., 1997; Wang and Struhl, 2004). We also observe autonomous inhibition of Notch activation in posterior compartment cells that overexpress Dl (*UAS:Dl*) with *en:GAL4* (*en∷Dl*), as measured by decreased Ct protein expression. Thus, the increased levels of Dl protein we observe in *en∷msk* wing discs may also explain the decrease in Ct protein expression in these wing pouches. However, we also observe increased Ct protein expression in posterior/dorsal cells when we overexpress both exogenous Msk and exogenous Dl simultaneously. What can explain these apparently paradoxical results?

We know that the ectopic Dl protein induced by Msk overexpression in wings is unable to signal to adjacent cells. However, if this ectopic Dl expression is sufficient to autonomously inhibit Notch signaling in these cells (as observed by a decrease in Ct protein expression), it may function in a dominant-negative fashion in some cells but not in others. Thus, when we co-express both Msk and Dl, two things happen. First, we are expressing functional Dl protein that is competent to signal to adjacent cells (*UAS:Dl*). Second, we are expressing non-functional Dl protein that is not competent to signal to adjacent cells, but is capable of autonomously inhibiting competent Dl protein (*UAS:msk*). There would then exist a situation within these cells where these two forms of Dl could compete for function. In those cells where competent Dl (*UAS:Dl*) wins, Ct expression is inhibited. In those cells where non-competent Dl (*UAS:msk*) wins, Ct expression can then be induced by competent Dl (*UAS:Dl*) expression in adjacent cells. This could account for the spotty appearance of Ct protein expression we observe in these discs (Figs. 4E, F).

Egfr, Msk, Delta, and MAPK nuclear translocation

We have shown that Egfr levels are decreased in *msk* clones in both larval wings and eyes, while Egfr levels are increased when we overexpress Msk in larval tissues. These data suggest the possibility of a regulatory feedback mechanism on Egfr protein expression in this tissue. Thus, in cells where MAPK can move into the nucleus, the initial activation of the Egfr/Ras/MAPK pathway leads to the nuclear translocation of MAPK in these cells, which subsequently results in further upregulation of Egfr levels in those cells. This increased Egfr expression then further promotes even greater MAPK nuclear translocation in those cells. In cells where pMAPK is held in the cytoplasm, Egfr levels are decreased, and this may act as a feedback signal for continued hold of pMAPK within the cytoplasm of these cells. Indeed, it has been previously reported that *Egfr* mRNA expression is reduced in developing pupal wings after hyper-activation of Egfr signaling by *rhomboid* (*rho*) overexpression

(*rho* encodes a protease required to activate the positive ligand *spitz*) (Sturtevant et al., 1994). The pMAPK induced by *rho* overexpression in developing pupal wings is also predominantly cytoplasmic, and leads to extra wing vein formation (Marenda et al., 2006). Thus, the regulation of Egf receptor levels may be a mechanism by which subsequent MAPK subcellular localization is controlled.

How could the subcellular localization of MAPK relate to Dl expression and function in developing *Drosophila* tissues? In clones of *spitz* (which encodes for an activating ligand for the Egfr pathway) Dl expression is lost in the developing eye (Tsuda et al., 2002). Similarly, clones of cells mutant for the Egfr receptor itself show a loss of Dl expression in the developing pupal eye, although these clones show normal Cut protein expression (Nagaraj and Banerjee, 2007). In the developing larval and pupal wing discs, *Dl* mRNA expression is absent in wing tissue double mutant for both *rhomboid* and *vein* (which effectively eliminates both the Egfr activating ligands *spitz* and *vein* in this tissue) (de Celis et al., 1997). Thus, Egfr activation and signaling are clearly required for Dl expression in these developing *Drosophila* tissues.

Dl expression is not lost in *msk* clones, suggesting: (1) the nuclear translocation of pMAPK is not required for Dl expression, (2) there is a redundant pMAPK nuclear transporter capable of importing pMAPK in these cells, (3) there is sufficient pMAPK nuclear translocation even in the absence of Msk protein to allow Dl expression to occur. Indeed, we have previously reported that *msk* null clones posterior to the morphogenetic furrow in the developing eye retain many important Egfr/Ras pathway functions (Vrailas et al., 2006). Yet, overexpression of Msk increases both Dl protein expression and *Dl* transcription, suggesting that the nuclear translocation of pMAPK is at least sufficient to increase Dl protein levels. However, the Dl induced by Msk overexpression is not competent to activate Notch signaling in adjacent cells, suggesting that the nuclear translocation of pMAPK alone is not sufficient to induce Notch signaling in adjacent cells. In wild-type wing cells, where we do observe high levels of competent, active Dl protein expression, we also observe high levels of phosphorylated, cytoplasmic MAPK, and low levels of Egfr protein expression. Similarly, where we observe high levels of Notch expression, we also observe high levels of Egfr protein expression. Gain-of-function mutations in *Notch*, or hyper-activation of the downstream Notch protein Enhancer of split (E(spl)) decrease *rho* expression, while loss-of-function mutations in *Notch*, or expression of a dominant-negative form of Notch increases *rho* expression and induces extra vein formation (de Celis et al., 1997; Sotillos and De Celis, 2005). pMAPK expression is also lost upon loss of *rho* expression (Guichard et al., 1999). Thus, Notch signaling represses pMAPK expression (Shilo, 2005). As the pMAPK expression induced by *rho* signaling is predominantly cytoplasmic (Marenda et al., 2006), we suggest that it may be the cytoplasmic hold of pMAPK that is normally required for Dl protein signaling competence to activate Notch in adjacent cells. When we overexpress competent Dl protein in the posterior compartment of developing wings (*en:Gal4*, *UAS:Dl*), we induce Notch activation in adjacent anterior/dorsal cells, and also induce increased expression of pMAPK in the posterior compartment. We have previously shown that pMAPK expression is lost in the posterior compartment of *en::msk* developing wing discs, as this pMAPK is ectopically translocated to the nucleus (Marenda et al., 2006). If pMAPK expression is required to induce Dl signaling competency, the difference in pMAPK expression observed between these two genotypes (*en::msk* and *en::Dl*) may explain the differences in Ct expression observed within these different genotypes as well.

Understanding how diverse signaling pathways integrate to regulate important biological processes is central to our understanding of the mechanisms of development. We are just recently beginning to understand these basic mechanisms of regulation, and how they function to coordinately control different cellular processes. In this report, we suggest that the subcellular localization of one pathway component (MAPK) as mediated by the nuclear import cofactor Msk is an important factor in Egfr signal regulation through the control of the expression of the Egfr protein itself. We further suggest that MAPK subcellular localization also plays an important role in the cross-talk between Egfr and Notch signaling pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.06.011.

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